

Cutting Edge: Innate Immune System Discriminates between RNA Containing Bacterial versus Eukaryotic Structural Features That Prime for High-Level IL-12 Secretion by Dendritic Cells¹

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RNA derived from bacterial but not eukaryotic sources, when transfected into human monocyte-derived dendritic cell precursors, induces high-level IL-12 secretion in conjunction with dendritic cell maturation stimuli. In vitro-transcribed mRNA that mimics the structure of bacterial mRNA in the lack of a long 3'-poly(A) tail likewise induces IL-12 secretion, but this property is lost upon efficient enzymatic 3'-polyadenylation. Among other tested RNAs, only polyuridylic acid induced IL-12 p70. This RNA response phenomenon appears biologically distinct from the classically defined response to dsRNA. RNA-transfected APC also polarize T cells in an IL-12-dependent manner toward the IFN- γ ^{high}IL-5^{low} Th1 phenotype, suggesting a link between the detection of appropriately structured RNA and the skewing of immune responses toward those best suited for controlling intracellular microbes. RNA structured to emulate bacterial patterns constitutes a novel vaccine strategy to engender polarized Th1-type immune responses. The Journal of Immunology, 2004, 172: 3989–3993.

The immune system requires means for distinguishing self tissues from infectious nonself, and mechanisms have evolved for identifying structures unique to potential pathogens. Such structures, termed pathogen-associated molecular patterns (PAMPs)³ (1), include bacterial cell wall components LPS and peptidoglycan, as well as nonmethylated CpG motifs in prokaryotic DNA.

RNA can also form PAMPs. A replicatory intermediate for some viruses, dsRNA provokes a characteristic type I IFN response (2, 3). Bacterial RNA might also contain PAMPs, because its ribosomal RNA differs from the eukaryotic in degree of methylation and pseudouridine content (4). Bacterial mRNA

also have absent or relatively short 3'-poly(A) tails (14–60 nt) compared with their eukaryotic counterparts, which almost always have long 3'-poly(A) tails (80–200 nt) (5, 6).

We hypothesized that the structural disparities between microbial and host RNA would have supplied selective pressure on the mammalian immune system to develop means to detect such RNA and modify responses accordingly. To test this hypothesis, we transfected RNA from several microbial species into monocyte-derived dendritic cell (DC) precursors, so that the RNA would reach intracellular locations. We then supplied the transfected cells with additional maturation signals and tested for the secretion of IL-12 p70. We focused on IL-12, because this cytokine plays an important role for the control of intracellular pathogens by skewing T cell responses toward the IFN- γ ^{high}IL-5^{low} Th1 phenotype (7). The secreted IFN- γ in turn confers upon macrophages the enhanced capacity to kill internalized microbes (8). We found that RNA from each of several bacterial species tested (but not eukaryotic RNA) was capable of priming DCs for robust IL-12 secretion upon maturation. In this study, we provide evidence that lack of long 3'-poly(A) tails, characteristic of bacterial mRNA, is one feature by which the innate immune system discriminates between RNA delivered to intracellular locations, and sets in motion immune responses modified to deal optimally with intracellular pathogens.

Materials and Methods

Preparation of RNA

Total RNA from *Streptococcus pyogenes* and *Enterococcus faecalis* was a kind gift from Dr. T. Buttaro (Temple University, Philadelphia, PA) and was prepared from mid-log growth cultures by the hot phenol technique. *Plasmodium falciparum* blood stage malaria parasites were a kind gift from Dr. N. Kumar (Johns Hopkins University, Baltimore, MD), with parasite RNA prepared using TRIzol extraction according to manufacturer's protocol (Life Technologies, Gaithersburg, MD). *Escherichia coli* RNA was prepared from mid-log growth bacteria by lysozyme digestion and subsequent RNAwiz extraction (Ambion,

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³ Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; DC, dendritic cell; pU, polyuridylic acid; pG, polyguanylic acid; pC, polycytidylic acid; pA, polyadenylic acid; p(I:C), polyinosinic-polycytidylic acid; SOD-1, superoxide dismutase 1; MART-1, melanoma Ag recognized by T cells 1; CD40L, CD40 ligand; CMM, cytokine maturation mixture; int, intermediate; pos, positive; neg, negative.

Austin, TX) according to the manufacturer's protocol. *Listeria monocytogenes* was a kind gift from Dr. Y. Patterson (University of Pennsylvania) with RNA likewise prepared using RNAwiz. RNA was quantified, and quality was assessed by spectrophotometry and agarose gel electrophoresis. Total RNA from human KG-1 leukemia line and mouse heart was purchased from Ambion. Purified poly(A)⁺ mRNA from mouse heart and mouse brain, as well as yeast tRNA, sheared salmon sperm DNA, and the homopolynucleotides polyuridylic acid (pU), polyguanylic acid (pG), polycytidylic acid (pC), polyadenylic acid (pA), and polyinosinic-polycytidylic acid (p(I:C)) were purchased from Sigma-Aldrich (St. Louis, MO). All RNAs were stored in siliconized tubes at -70°C .

In vitro-transcribed mRNAs, capMART-A₂₀ and capSOD-A₃₀, were prepared from linearized pT7TS-SOD-1 (Dr. K. Mitchell (National Institute of Dental and Craniofacial Research, Bethesda, MD)) and pT7T3D-MART-1 (American Type Culture Collection, Manassas, VA) plasmids encoding rat superoxide dismutase 1 (SOD-1) and human melanoma Ag recognized by T cells 1 (MART-1), respectively. These transcripts, generated using MessageMachine Ultra (Ambion), had short 20- and 30-nt-long poly(A)-tails and cap structure as indicated. Selected aliquots of capSOD-A₃₀ and capMART-A₂₀ were polyadenylated with *E. coli* poly(A) polymerase (Ambion) to yield capSOD-A_{>150} and capMART-A_{>150} mRNAs with a minimum of 150-nt-long poly(A) tail.

When indicated, plus- or minus-strand MART mRNAs were used. These RNAs were noncapped, generated by MegaScript kits (Ambion) from pT7T3D-MART-1 using the corresponding T7 or T3 RNA polymerase. This kit was also used to transcribe another noncapped mRNA (designated as luc) from firefly luciferase-encoding plasmid pluc (Dr. D. Gallie (University of California, Riverside, CA)). Transcribed mRNAs were precipitated using LiCl, washed with ice-cold 70% ethanol, and resuspended in RNase-free water, and concentration and quality were assayed by spectrophotometry and agarose gel electrophoresis.

To promote duplex formation of in vitro-transcribed complementary positive- and negative-strand RNA, $\sim 1 \mu\text{g/ml}$ each RNA were mixed and incubated at 37°C for 3 h, cooled to 4°C for 1 h, and frozen at -70°C before use. A similar procedure was used for synthetic polyribonucleotide inhibition of IL-12 induction by capMART-A₂₀ using graded concentrations of pA and p(A,C,U), with the exception that these preparations were used immediately without prior freezing.

Preparation of human PBMC fractions

Healthy adult donors provided informed consent and were leukapheresed under an Institutional Review Board-approved protocol. Blood product was then elutriated to obtain monocyte-rich (>94%) and lymphocyte-enriched fractions that were cryopreserved as described previously (9).

DC culture and transfection

DC culture schemes were modified from rapid 2-day methods described previously using either CD40 ligand (CD40L) or LPS as primary drivers of maturation (9, 10). Briefly, cells from monocyte-rich elutriation fractions were cultured overnight at 1.5×10^6 cells/ml in macrophage serum-free medium (Life Technologies) supplemented with 50 ng/ml GM-CSF (for cells to be matured with CD40L (Amgen, Thousand Oaks, CA)) or GM-CSF plus 1000 U/ml IL-4 (for cells to be matured with *E. coli* O26:B6 LPS (Sigma-Aldrich)). Total volume for 48-well tissue culture plates was 1 ml, and for 96-well plates, 0.2 ml. The next day, culture supernatants were removed, and cells were transfected using lipofectin (Life Technologies) with indicated concentrations of RNA as previously described (11) for 45 min. When noted, aliquots of nucleic acids were incubated with Benzonase (Novagen, Madison, WI) or DNase I (Ambion) before complexation. After this period, supernatants were removed and replaced with fresh medium and cytokines. After 1–3 h, a maturation stimulus in the form of $1 \mu\text{g/ml}$ CD40L (Amgen) or 50 ng/ml LPS O26:B6 (Sigma-Aldrich) was added to cultures. Twenty-four hours later, supernatants were removed and assayed for the presence of cytokines. In some experiments, a cytokine maturation mixture (CMM) consisting of TNF- α , PGE₂, IL-6, and IL-1 β was used to mature DCs as untransfected standards of comparison according to published methods (12, 13). In general, before maturation, DC cultures were somewhat heterogeneous (intermediate-negative) in CD14 expression and CD83^{neg}, CD80^{low}, CD86^{low}, CD11c^{pos}, HLA-DR^{int}, HLA class I^{pos}, and CD33^{pos}. After maturation, cells were CD14^{low-neg}, CD83^{pos}, CD80^{high}, CD86^{high}, CD11c^{pos}, HLA-DR^{high}, HLA-class I^{pos}, and CD33^{pos}.

ELISA

Capture and biotinylated detection Abs and standards for human IFN- γ , IL-5, and IL-12 p70 (BD PharMingen, San Diego, CA), were used according to the manufacturer's recommendations and protocols as described previously (9). IFN- α was measured with an ELISA system that detects the most common isoforms of IFN- α (Biosource, Camarillo, CA), according to the manufacturer's instructions.

Allosensitization of T cells

Naive CD4⁺CD45RO⁻ T cells were prepared from lymphocyte-rich elutriation fractions using negative depletion columns as directed by the manufacturer (R&D Systems, Minneapolis, MN). Purified allogenic T cells (1×10^6 /well) were cocultured as described previously with DCs (1×10^7 /well) that were collected 12–14 h after maturation stimulus (just before peak IL-12 secretion) in 48-well tissue culture plates. On day 6, the T cells were harvested and restimulated on plates coated with anti-CD3 and anti-CD28 as described previously (9). In some experiments, cocultures were performed in the presence of $10 \mu\text{g/ml}$ IL-12-neutralizing mAb or isotype-matched negative control (R&D Systems). Supernatants were harvested 24 h later and analyzed by ELISA.

Results and Discussion

Bacterial RNA and in vitro-transcribed mRNA prime DC for high-level IL-12 secretion

We hypothesized that agents of the innate immune system could distinguish between RNA of mammalian and microbial origin. Therefore, we transfected monocyte-derived DC precursors with total (prokaryotic) RNA from *S. pyogenes*, the etiologic agent of scarlet fever, as well as total (eukaryotic) RNA isolated from the human leukemia line KG-1, mouse heart, and the human malaria parasite *P. falciparum*. Cells were then either matured or not with CD40L, and culture supernatants were analyzed 24 h later for IL-12. Cells treated with either CD40L or RNA alone produced little or no IL-12 (not shown). However, cells transfected with *S. pyogenes* RNA and then matured secreted very high levels of IL-12 (Fig. 1A). The other RNA had no such effect. In contrast, RNA from *E. coli*, *E. faecalis*, and *L.*

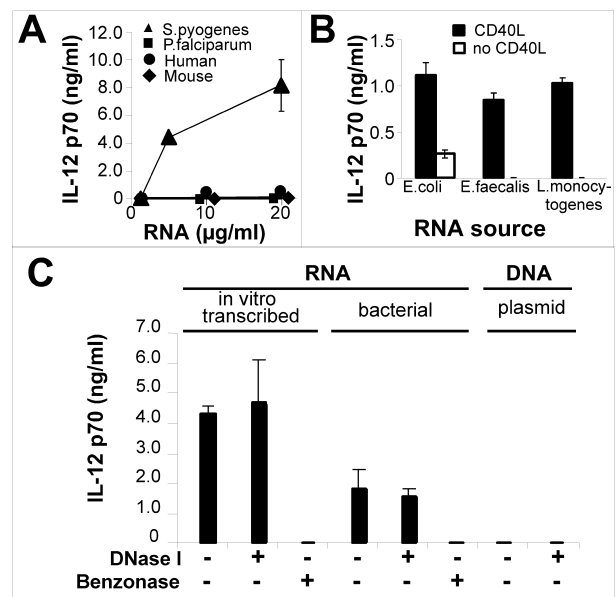


FIGURE 1. Bacterial RNA primes for high-level IL-12 secretion by DC. *A*, Overnight cultures of monocyte-derived DC precursors were transfected with graded doses of total RNA from *S. pyogenes*, *P. falciparum*, human leukemia line KG-1, or mouse heart, and then matured with $1 \mu\text{g/ml}$ CD40L. Supernatants were collected 24 h later and tested for IL-12 p70 by ELISA. *B*, Transfection with total RNA from *E. coli*, *E. faecalis*, or *L. monocytogenes* at a concentration of $5 \mu\text{g/ml}$, followed by maturation with CD40L or no treatment, with 24-h supernatants assessed for IL-12 p70. *C*, In vitro-transcribed capSOD-1 A₃₀ RNA, total *E. coli* RNA, and bacterial plasmid DNA encoding MART-1 Ag were digested with DNase I or Benzonase, and transfected into DC precursors at $5 \mu\text{g/ml}$ based on predigestion concentration of RNA. Transfected cells were matured with CD40L, and supernatants were assessed for IL-12 as in *B*. Results in *A–C* are each representative of three experiments with different donors \pm SEM.

monocytogenes, the etiologic agent of listeriosis, each induced high-level IL-12 secretion in conjunction with CD40L treatment (Fig. 1B).

Maximization of IL-12 secretion by maturing DCs is tightly regulated and requires two signals, including IFN- γ (priming signal, or signal 1) followed by CD40L or LPS (signal 2) (14). These results show that RNA substitutes for IFN- γ as the first (i.e., priming) signal. It should be noted here that RNA did not appreciably alter the surface phenotype of DCs (i.e., CD83, CD80, and CD86) as assessed by flow cytometry, and did not induce detectable IFN- γ , nor could RNA serve as the second signal after exogenous IFN- γ priming (data not shown). Furthermore, transfection rather than mere addition of RNA was required to observe IL-12-priming effects (not shown). This seems to indicate that intracellular delivery of RNA is required, although a mere stabilizing effect of the lipofectin complex cannot be ruled out. Perhaps more importantly, all tested bacterial RNA primed for IL-12 secretion, whether derived from pathogenic or generally harmless species, Gram-negative or Gram-positive species, or species that cause disease through either intracellular or extracellular lifestyles. It thus seemed that the discrimination, based on the set of RNA examined, cut exclusively along eukaryotic/prokaryotic boundaries.

To show that the observed effects were actually triggered by RNA rather than some contaminant, *E. coli* RNA was subjected to enzymatic action by either Benzonase, which digests both DNA and RNA, or RNase-free DNase I. Electrophoretic analysis showed Benzonase destruction of *E. coli* RNA, as well as in vitro-transcribed RNA (capSOD-1 A₃₀) and MART-1 DNA plasmid controls (not shown). DNase I only destroyed plasmid DNA and had no apparent effect on RNA. As expected, Benzonase-treated, but not DNase-treated *E. coli* RNA lost the capacity to prime DC for IL-12 secretion, consistent with RNA being the active agent (Fig. 1C).

3'-end polyadenylation of in vitro-transcribed mRNA inhibits capacity to prime for IL-12 secretion

The previous results also showed that in vitro-transcribed capSOD-1 A₃₀ mRNA, like natural bacterial RNA, had the capacity to prime DCs for IL-12 secretion (Fig. 1C). We therefore tested a battery of nucleic acids, both natural and in vitro synthesized, to discover whether any other IL-12-priming RNAs existed, and if so, what structural similarities tied them to bacterial RNA. DCs were transfected with in vitro-transcribed mRNA encoding firefly luciferase, poly(A)⁺ rodent mRNA from various tissues, yeast tRNA, sheared salmon sperm DNA, as well as homopolyribonucleotides pA, pU, pC, and pG, and the random polymer p(A,C,U). Cells were then treated with CD40L or bacterial LPS, and 24-h supernatants were tested for IL-12 (Fig. 2A). Luc RNA primed for robust IL-12 secretion whether matured with CD40L or LPS. However, no other tested RNA had such activity except pU. The finding with pU was initially surprising, because we could see no particular connection with bacterial RNA. In contrast, we appreciated at once that in vitro-synthesized RNA, like bacterial mRNA, had either very short or absent 3'-poly(A) tails compared with natural mammalian mRNA (5, 6), which in these experiments did not induce IL-12. This disparity in poly(A) tail length might therefore serve as a basis of discrimination.

We tested this hypothesis by enzymatically extending the 3'-poly(A) tail of in vitro-synthesized mRNA encoding SOD-1

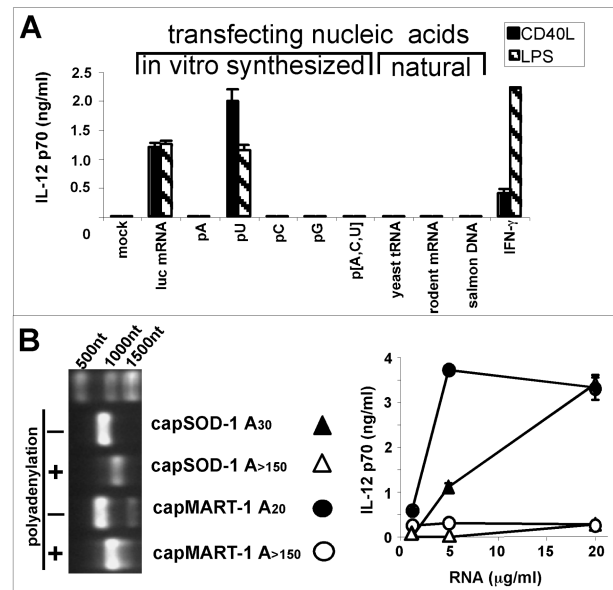


FIGURE 2. mRNA with short or absent 3'-poly(A) tail and pU primes DCs for IL-12 secretion. *A*, Overnight cultures of DC precursors were transfected with indicated in vitro-synthesized or natural-source nucleic acids at 5 μ g/ml and then matured with either CD40L or bacterial LPS, with culture supernatants collected 24 h later and assayed for IL-12 p70. Results are representative of two separate experiments. *B*, In vitro-transcribed SOD-1 and MART-1 mRNA were subjected to enzymatic polyadenylation that extended their 3'-poly(A) tail by at least 150 nt as indicated by 1.2% agarose gel electrophoresis. These RNAs were then transfected into DC precursors at increasing concentrations, and cells were matured with 1 μ g/ml CD40L. Culture supernatants were collected 24 h later and analyzed by ELISA for IL-12 p70. Results are representative of three experiments.

(capSOD-1 A_{>150}) and the human melanoma-associated Ag MART-1 (capMART-1 A_{>150}) (Fig. 2B). These mRNAs were then transfected into DCs at graded concentrations and compared with their short-tailed parental transcripts (capSOD-1 A₃₀ and capMART-1 A₂₀) for priming IL-12 secretion. IL-12 secretion was strongly diminished for enzymatically polyadenylated RNA across the tested dose range. Therefore, poly(A) tail length appears to serve as a means of distinguishing between endogenous mRNA and RNAs that could be of microbial pathogen origin.

Response of DC to IL-12-priming mRNA is distinct from the classic response to dsRNA

The classical type I IFN response is induced by dsRNA (2, 3). Although our RNA constructs are nominally single-stranded, limited internal sequence complementarity could form short regions of double-strandedness sufficient to induce a type I response and possible cosecretion of IL-12. Therefore, we tested the capacity of p(I:C), the synthetic model dsRNA, to prime for both IL-12 and IFN- α secretion as compared with pU, and p(A,C,U). Only supernatants from cells transfected with pU showed high levels of IL-12 (Fig. 3A). In contrast, p(I:C) was the most potent IFN- α inducer, with pU and p(A,C,U) stimulating significant, yet more modest, levels.

In related experiments, the IL-12-priming capacity of dsRNA, formed from in vitro-synthesized complementary ssRNA was tested. Both pU and capMART-1 A₂₀ mRNA (which both prime for IL-12) were individually incubated with increasing concentrations of pA or p(A,C,U) (which both do not

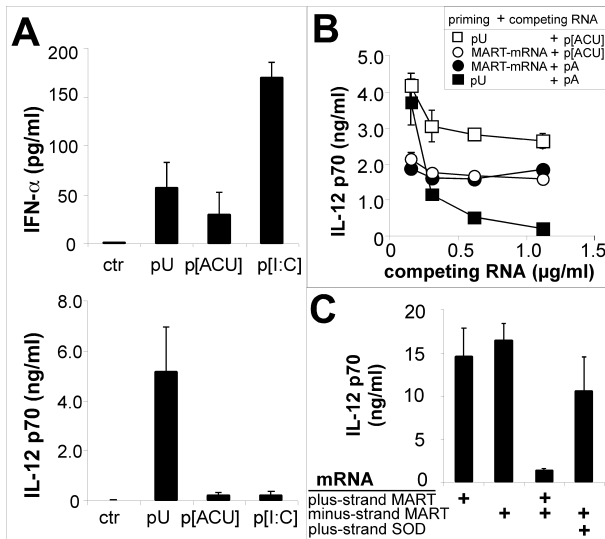


FIGURE 3. ssRNA primes for IL-12 secretion through a pathway distinct from those activated by dsRNA. DC precursors were transfected with 5 μg/ml polyribonucleotides pU, p(A,C,U), or p(I:C) (A), or with 5 μg/ml pU or MART-1 RNA that were coincubated with graded doses of inhibitor polyribonucleotides p(A,C,U) or pA before the formation of lipofectin complexes (B), or with 5 μg/ml MART-1 plus-strand RNA, MART-1 minus-strand, MART-1 minus-strand plus plus-strand, or MART-1 minus-strand plus SOD-1 plus strand. Cells were then matured with CD40L (A) or LPS (B and C). After 24-h incubation, culture supernatants were removed and assayed for IL-12 p70 and IFN-α. Results of A were averaged from four experiments with different donors, and B and C are representative of three experiments, ± SEM.

prime for IL-12) before the formation of lipofectin complexes. Under these conditions, it would be expected that pA would hybridize with pU to form double strands, due to perfect complementarity in base pairing. Such interactions should not occur efficiently between p(A,C,U) and pU. In contrast, neither pA nor p(A,C,U) should form significant duplexes with capMART-1 A₂₀. When these mixtures were transfected into DC, it was found that the presence of pA strongly inhibited IL-12 priming by pU in a dose-dependent manner (Fig. 3B). Any inhibition by p(A,C,U) on pU was mild and incomplete. In contrast, neither pA nor p(A,C,U) inhibited the capacity of capMART-1 A₂₀ to prime for IL-12 secretion at the same doses, suggesting that the suppressive effects on pU were specific at these concentrations, and likely a result of the formation of inactive (with respect to IL-12 priming) dsRNA. Similarly, when in vitro-transcribed, complementary MART-1 plus- and minus-strands were coincubated before transfection, the IL-12-priming properties possessed by either strand alone were strongly inhibited (Fig. 3C). In contrast, coincubation of MART-1 minus-strand with SOD-1 plus-strand did not appreciably diminish IL-12 priming, presumably due to the lack of complementary sequences between the two strands. These data are consistent with the notion that the IL-12 priming response induced by ssRNA is phenomenologically distinct from the type I IFN response induced by dsRNA, and therefore represents a distinct and probably novel PAMP recognition system.

DCs transfected with mRNA that prime for IL-12 secretion polarize naive allogenic CD4⁺ T cells toward Th1

Finally, we tested the capacity of RNA-transfected DC to polarize CD4⁺ T cells toward Th1 phenotype in an allosensitiza-

tion assay that measured cytokine secretion (9). CD40L-matured DCs previously transfected with pU, capSOD-1 A₃₀, or *S. pyogenes* total RNA (all inducing IL-12 secretion) sensitized T cells that were strongly polarized toward Th1 type as evidenced by their high IFN-γ secretion with correspondingly lower capacity to secrete IL-5 (Fig. 4A). In contrast, DCs transfected with the RNA that do not prime for IL-12 secretion (p(A,C,U), capSOD-1 A_{>150}, and KG-1 human RNA) sensitized T cells that secreted less IFN-γ but enhanced IL-5, characteristic of Th2. This is the same general cytokine secretion profile induced by untransfected CMM-induced standard DC (which likewise do not secrete IL-12 p70) that we used for comparison (12, 13). The RNA-induced Th1 polarization could be blocked by IL-12-neutralizing mAb (Fig. 4B). These results show that RNAs containing the appropriate structure can prime DC for IL-12 secretion at a magnitude sufficient to strongly polarize T cells toward the Th1 phenotype.

The results of these studies show that cellular components of the innate immune system can distinguish between bacterial and eukaryotic RNA through a mechanism that leads to high-level secretion of IL-12, an enabler of strong Th1-type immunity critical for controlling intracellular pathogens. This discrimination could be recreated using in vitro-transcribed RNA that mimicked bacterial or eukaryotic transcripts by virtue of either short or long 3'-poly(A) tails, respectively. However, other structural features may play a role and are currently under investigation. The action of pU remains obscure, because we could not relate pU to bacterial mRNA structure. The pU could possibly act indirectly, by hybridizing with the 3'-poly(A) tails of endogenously produced cellular mRNA, masking the tail and making it appear to the putative RNA recognition apparatus as bacterial in origin. Alternatively, a separate, direct mechanism for identifying pU may have evolved as a means for detecting the genomic RNA of some minus-strand RNA viruses, which

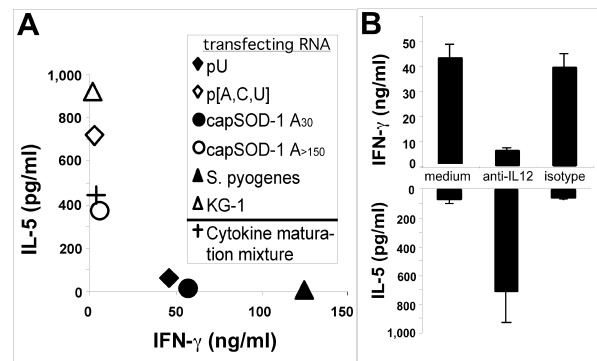


FIGURE 4. Transfection with RNA that primes for high-level IL-12 secretion allows DCs to functionally polarize naive CD4⁺ T cells toward Th1 phenotype. A, DC precursors were transfected with 5 μg/ml pU, p(A,C,U), capSOD A₃₀, capSOD A_{>150}, *S. pyogenes* total RNA, or KG-1 total RNA. Cells were then matured with CD40L and harvested 14 h later, before their peak secretion of IL-12. DCs were cocultured with column-purified CD4⁺CD45RO⁺ naive allogenic T cells. For comparison, cocultures with untransfected monocyte-derived DC prepared using a standard CMM were also initiated. After 6 days, T cells were harvested and restimulated in plates pre-coated with anti-CD3 and anti-CD28 Abs. After 24-h incubation, culture supernatants were harvested and assayed for IFN-γ and IL-5 by ELISA. B, ELISA analysis of supernatants from restimulated T cells sensitized in the presence of IL-12-neutralizing or isotype-matched control mAb by DCs transfected with *S. pyogenes* RNA. Results in A and B are representative of three experiments with different donors ± SEM.

encode the poly(A) tails of their mRNA through long stretches of poly(U). These two possibilities are currently under investigation. Thus, we have identified novel RNA-associated PAMPs that shed light on a previously undescribed mode of discrimination by the innate immune system. These PAMPs may have implications for natural microbial immunity and autoimmunity as well as practical application in the formulation of RNA-based vaccines or a new class of RNA-based adjuvants using prokaryotized structural features that could optimize cell-mediated immunity and Th1 polarization for controlling infectious diseases or cancer.

Note added in proof: Recent work by others (15, 16) has suggested that ssRNA from viruses as well as some synthetic RNA constructs may be recognized in mice by Toll-like receptor 7 and in humans by Toll-like receptor 8.

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